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GENETIC STUDIES OF PIGMENTATION IN STAPHYLOCOCCUS AUREUS

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May 1964

ABSTRACT

Exposure of cells of several strains of Staphylococcus aureus to 50 or 100 μg of N-methyl-N'-nitro-N nitrosoguanidine for 30 to 60 minutes induced large numbers of mutants with pigment content different from that of the parent. By determination of the amount of pigment as related to the optical density of the cells, four to seven classes of pigmentation mutants could be defined. Mutants with pigment content differing from that of the parent could be readily mutated to other pigmentation states and are thus probable point mutations. In contrast, completely white mutants could not be induced by the mutagen to any degree of pigmentation and possibly represent minor deletions in the chromosome. There was no evidence that the pigment gene(s) is extrachromosomal. Mutants differing in pigment content from that of the parent were unable to produce coagulase during growth although the parent cultures elaborated considerable coagulase under identical conditions.

I. INTRODUCTION

The differing degrees of pigmentation of various isolates of Staphylococcus aureus and the occurrence of occasional variants of lesser pigmentation in many cultures of these organisms have been recognized for some time. Systematic analysis of the genetics of the pigmentation character has apparently not been attempted and recent reviews[1,2] have offered few facts bearing on this subject. The potent mutagenic activity of nitrosoguanidine (N-methyl-N'-nitro-N-nitrosoguanidine) documented for various gram-negative organisms[3,4] provided an opportunity to study this genetic trait without the necessity for selective media to detect the resulting mutants. In the present study, data are presented to show the number and kind of induced pigmentation mutants in several strains of S. aureus and conclusions are drawn concerning the nature of these mutational changes.

II. METHODS

The strains of S. aureus employed were those isolated from clinical material submitted for bacteriological analysis to the laboratory of the Frederick Memorial Hospital, Frederick, Maryland. These isolates were labeled by number or patient's name and are not related to other commonly used strains. Stock cultures were maintained on trypticase soy agar at 4 C and transferred every 4 to 6 months.

Cells were exposed to the mutagenic activity of nitrosoguanidine (N-methyl-N'-nitro-N-nitrosoguanidine, Aldrich Chemical Co., Milwaukee, Wis.) by the following method. Tubes containing 5 ml of trypticase soy broth (Baltimore Biological Laboratory) were inoculated with 0.05 ml of a 1:10 dilution of overnight broth culture of the strain to be employed. Following 4 hours of incubation on a shaker at 37 C, the cells were sedimented by centrifugation (1640 x g for 15 minutes) and resuspended in an equal volume of sterile 0.85% saline. A sufficient volume of sterile saline solution of nitrosoguanidine was added to give the desired final concentration, 50 or 100 μg per ml. The suspensions were placed on a shaker at 37 C and sampled at appropriate intervals for up to 60 minutes. Each sample was diluted in saline and plated by spreading on the surface of previously poured trypticase soy agar plates. These plates were then incubated at 37 C for 24 hours followed by incubation at room temperature for 48 hours to intensify pigmentation. They were then scored for numbers of mutants and for total viable count. Pigmentation mutants from plates were transferred to trypticase soy agar slants. Nitrosoguanidine solutions were prepared by dissolving the drug at a concentration of 1 mg per ml in saline at 45 C. After the drug had dissolved, the solution was sterilized by filtration through a sintered glass filter. Mutagen solutions are rather unstable and must be prepared daily for reproducible results.

The following method was employed to determine pigment content of various clones. Overnight broth cultures of mutants were diluted in sterile broth and, for each mutant, two plates of trypticase soy agar were inoculated by spreading each plate with 0.1 ml of the 10^6 dilution. The plates were incubated at 37 C for 24 hours followed by 48 hours of incubation at room temperature. The cells from both plates were harvested by suspending in saline and the volume of the suspension was adjusted to 5 ml. The suspension was then diluted 1:50 in saline and the optical density of the dilution at 650 mμ was determined on a Coleman Junior spectrophotometer. The dense suspension of cells was then centrifuged for 15 minutes at 1640 x g, the supernatant fluid was discarded, and 5 ml of absolute methanol were added to each tube. The cells were then resuspended and the pigment was extracted by gentle boiling of the methanol for 10 minutes by immersing the tubes in a 75 C water bath. The volumes were adjusted to 5 ml by adding more methanol and the suspension was centrifuged for 20 minutes at 1640 x g. The supernatant was decanted into tubes and stoppered. The optical density of these methanol extracts containing the pigment was determined at 460 mμ, the absorption maximum on the Coleman Junior spectrophotometer. No attempt was made to distinguish between the hydrocarbon carotenoids and the carotenoid alcohols. The ratio of the optical density of the extracted pigment to the optical density of the 1:50 dilution of cell suspension was employed as a measure of the degree of pigmentation of the mutants. A similar method has been utilized by Wilson and Nunez[5] for their studies on the effects of nutrition and environment on pigmentation in S. aureus.

Coagulase production of various mutants and of the parent types was determined during growth in trypticase soy broth containing 0.3% bovine serum albumin. The detailed method has been presented elsewhere.[6]

III. RESULTS

A. KILLING AND PIGMENTATION MUTANT INDUCTION BY NITROSOGUANIDINE

Cells of the Maybush strain of S. aureus were exposed to concentrations of nitrosoguanidine ranging from 5 to 100 μg per ml. The suspensions were periodically diluted and plated according to the methods already described. Relatively low concentrations of the mutagen (5 or 10 μg per ml) exhibited little lethality nor were there many mutants of differing pigmentation in the treated population. With higher concentrations of the drug (20 to 100 μg per ml) there was an increasing rate of killing resulting in a higher proportion of pigmentation mutants among the survivors. The data in Figure 1 show the decline in viability and the rise in percentage of mutants in the survivors of cells exposed to 100 μg of the mutagen per ml. Similar results obtained with several other strains of S. aureus exposed to only 50 μg of

drug per ml are presented in Table 1. It is clear that these strains display roughly equivalent sensitivity to the mutagenic action of the drug. The white or completely nonpigmented mutants are easily counted but the various mutants of lesser pigmentation than the parent are difficult to enumerate.

B. MUTABILITY OF PIGMENTATION MUTANTS

Various clones of white or semipigmented mutants were treated with nitrosoguanidine and the viability and mutant induction were determined as before. Figure 2 shows that the white clones derived from the Maybush strain fail to show any mutants possessing pigment although the killing rate was equivalent to that obtained during exposure of the parent type to the same concentration of the mutagen. Ten white mutants from strain Maybush were exposed to 100 µg of nitrosoguanidine per ml for 60 minutes and none showed pigmented mutants although killing was extensive. A representative series of pigmentation mutants from strain Smith were exposed to nitrosoguanidine and the numbers of survivors showing pigmentation differing from the parent clone were scored. The data in Table 2 show that the white mutants were nonrevertible by this treatment, but other clones possessing some degree of pigmentation were easily mutated to pigmentation types other than that of the starting culture. Series of pigmentation mutants from several other strains showed identical behavior.

C. ATTEMPTS AT CURING AND CONTACT TRANSFER OF PIGMENTATION

The absence of induced back mutation to pigmentation in the white types and the readily demonstrable mutational reversal of semipigmented types strongly suggested that genes for pigmentation are irretrievably lost in the white mutants. The possibility that chromogenesis might be due to an extrachromosomal factor was tested by growth of the parent strains in media containing conventional curing agents for plasmid genes. Strains Smith and Maybush were both grown in broth containing from 0.1 to 50 µg/ml of acriflavin, acridine orange, or proflavin. Growth was allowed to continue up to 26 hours at 37 C. The cultures were diluted and plated after various times of incubation and the numbers of white or semipigmented mutants in the population were assessed. White mutants did appear after 18 or more hours of incubation. The frequency of white mutants was erratic, however, and varied from 0.01 to 0.3% of the total viable count. In addition, the numbers of white mutants did not follow decimal dilution patterns. Control platings from growth tubes not containing the drugs invariably were negative for pigmentation mutants. It is noteworthy that the mutants appearing in tubes containing curing drugs were almost always white or completely nonpigmented, and semipigmented clones were rarely observed. This point is of some significance and will be discussed later.

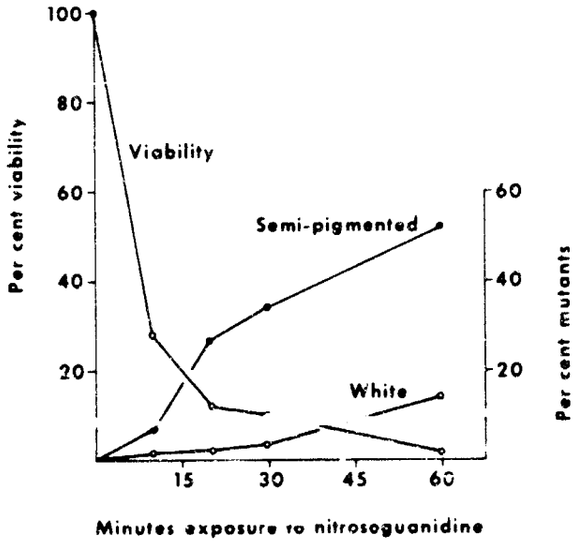

Figure 1. Death Rate and Pigment Mutant Production During Exposure of Maybush Strain of S. aureus to Nitrosoguanidine.

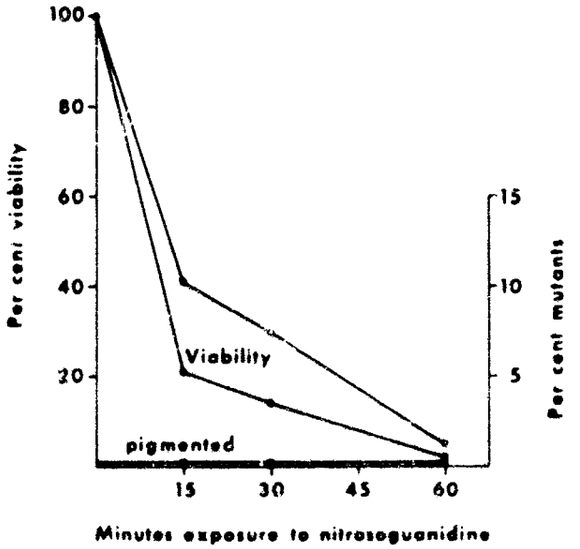

Figure 2. Death Rate and Absence of Back Mutation During Exposure of White Mutants of Maybush Strain to Nitrosoguanidine.

TABLE 1. YIELD OF WHITE MUTANTS FOLLOWING NITROSOGUANIDINE TREATMENT OF VARIOUS STRAINS OF S. AUREUS

Strain	Per Cent White Mutants After Treatment for 15 min	30 min	60 min
Pitman	0.6	1.5	3.1
Lovett	0.4	1.3	2.3
Smith	ND[a]	1.7	2.8
#3	ND	2.9	5.5

a. Not determined. All strains exposed to 50 μg nitrosoguanidine per ml for indicated time.

TABLE 2. NITROSOGUANIDINE-INDUCED[a] MUTATION OF PIGMENTATION VARIANTS OF SMITH STRAIN OF S. AUREUS

Mutant Class[b]	Per Cent Other Pigmentation Types
I	0
I	0
I	0
II	8.9
II	8.3
II	11.5
IV	1.8
V	33.0

a. Cells exposed to 50 μg of nitrosoguanidine for 60 minutes.

b. Mutant classes defined in Figure 3.

Numerous attempts were made to show contact transfer of pigmentation. Auxotrophic but heavily pigmented mutants were mixed in varying proportions and cell densities with 15 randomly selected mutants. After various times of incubation at 37 C, the mixed cultures were diluted and plated on minimal agar that supported growth of the white mutant but prevented growth of the pigmented auxotrophs. Under no conditions tested was pigmentation transferred to the prototrophic, white type.

D. CLASSES OF PIGMENTATION MUTANTS

A large number of surviving clones was picked from plates of strains Lovett and Smith exposed to 50 μg nitrosoguanidine per ml for 60 minutes and from plates of Maybush strain exposed for only 30 minutes to the same concentration of mutagen. These clones were checked for purity by streaking. The amount of pigment in cells of these clones as a function of cell density was then determined as described in Section II, Methods. The pigment content/cell density functions were then plotted as presented in Figure 3. It is clear from these data that there are distinct classes of pigmentation mutants and that there is not a continuous spectrum of mutant types. Unexpectedly, mutant classes possessing heavier pigmentation than the parent type arise after exposure to the mutagen for 60 minutes. There are more classes of mutants appearing in the strains exposed to mutagen for 60 minutes (Lovett and Smith strains) than in Maybush strain exposed for only 30 minutes. However, many of the survivors following mutagen treatment for 60 minutes have much slower growth rates and smaller colony sizes than the parent type and probably represent multiple mutants.

E. COAGULASE PRODUCTION BY PIGMENTATION MUTANTS

Related research in this laboratory on the kinetics of coagulase release by S. aureus prompted an examination of coagulase production by the classes of pigmentation mutants. Two representatives of each mutant class having a growth rate equivalent to the parent type were employed. The results presented in Table 3 show that, of the clones tested, none produced coagulase except those that possessed the same degree of pigmentation as the parent type and are presumably unaltered parent clones. The close genetic relationship between pigmentation and coagulase production observed here is somewhat more restrictive than has been previously recognized in general surveys of coagulase formation by isolates of the pigmented and nonpigmented variants of this organism.

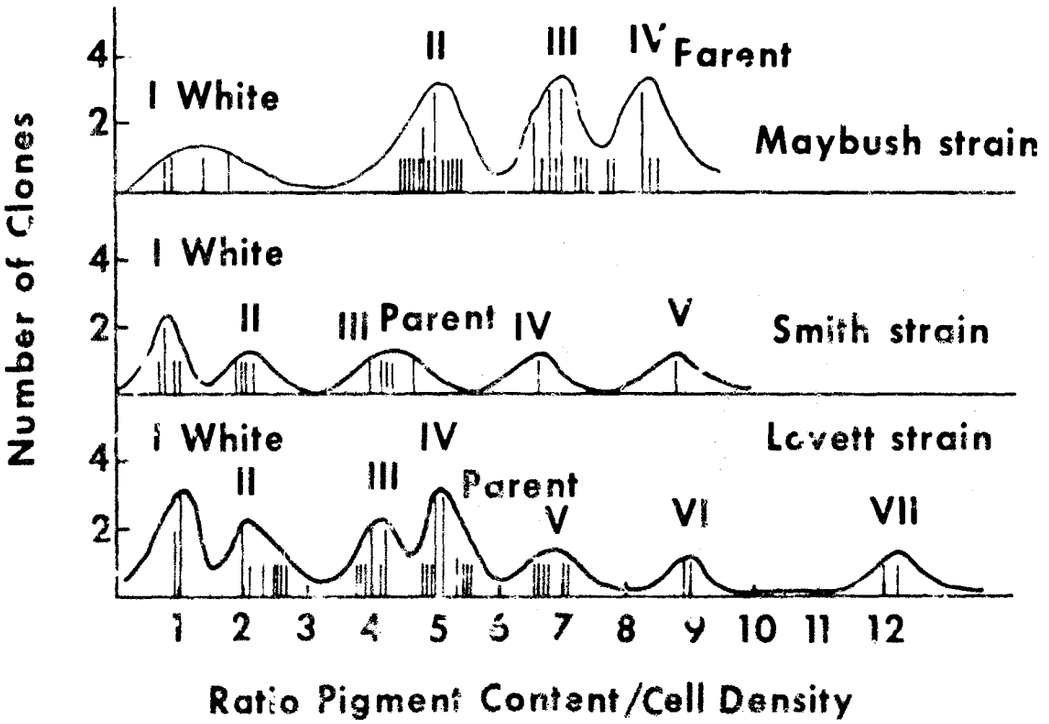

Figure 3. Classes of Pigmentation Mutants Obtained from Three Strains of S. aureus Following Exposure to Nitrosoguanidine.

TABLE 3. COAGULASE FORMATION BY MEMBERS OF CLASSES OF PIGMENTATION MUTANTS IN LOVETT STRAIN OF S. AUREUS

Mutant Class[a]	Units Coagulase per ml[b]
I	0
I	0
II	0
II	0
III	0
III	0
IV	3.4
IV	5.1
VI	0
VI	0
VII	0
VII	0

a. Mutant classes defined in Figure 3.
b. Coagulase produced during 80-minute incubation in trypticase soy broth plus 0.3% bovine serum albumin.

IV. DISCUSSION

The ready mutability of semipigmented clones to other degrees of pigmentation strongly suggests that these semipigmented mutants are point mutations in the pigment gene(s). The absence of any demonstrable back mutation in the white, nonpigmented types indicates a loss of the gene for pigmentation. Failure to obtain any convincing evidence of a possible extrachromosomal nature for the pigment gene supports the concept that pure white mutants of this organism represent minor chromosomal deletions. Acridine dyes are known to produce considerable numbers of small deletions as well as point mutations in bacteriophage,[7] a fact that is probably the basis for the predominance of white mutants appearing in cultures grown in the presence of these dyes. Efforts to determine whether semipigmented mutants were transition or transversion mutants have failed because of the relatively weak mutagenic activity of 5-bromodeoxyuridine, which specifically produces transition mutants.[7]

The present primitive understanding of the enzymology of pigment synthesis in S. aureus precludes a precise study of the alterations in pigment-synthesizing enzyme resulting from mutations in the pigment gene. On a purely empirical basis, it can be postulated that the mutants observed in this study involve the terminal enzyme in the process of pigment synthesis. Mutations throughout a hypothetical series of enzymes involved in sequential synthesis of the final pigment would be expected to give rise to a large number of classes of pigment mutants, but this has not been experimentally observed. The fact that only four to seven classes of mutants appeared may indicate either that comparatively few alterations in pigment gene structure result in an enzyme with modified properties or that there are mutational hot spots in the pigment gene. Resolution of this question would require genetic and biochemical techniques not yet developed for the pigment formation system.

Loss of formation of coagulase by the pigmentation mutants can not be adequately explained at present. Point mutations, as in the semipigmented mutants, would not be expected to alter transcription of the coagulase gene even if it were in the same operon. Consequently, lack of coagulase production in pigmentation mutants can be most conveniently regarded as an indirect or pleiotrophic effect of the mutation in the pigment gene.

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